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Award Number: W81XWH-07-1-0376

TITLE: New Conditionally Replicating Adenovirus Vectors for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Stephen Dewhurst, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14627

REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2009	Annual	1 Sept 2008 – 31 Aug 2009

4. TITLE AND SUBTITLE
New Conditionally Replicating Adenovirus Vectors for Breast Cancer Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER W81XWH-07-1-0376

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S): Stephen Dewhurst, Ph.D.

Email: stephen_dewhurst@urmc.rochester.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rochester
Rochester, NY 14627

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT – Enter a brief, 200 words, unclassified summary of the most significant finding during the research period.
There is a pressing need to develop new treatments for breast cancer. Oncolytic, conditionally-replicating adenoviruses (CRADs) represent one such approach. Our objective to develop new CRADs containing mutant DNA polymerases with a high functional dNTP requirement; we hypothesize that these vectors will replicate selectively in tumor cells. We have identified an important surrogate for the adenovirus DNA polymerase (Ad pol): the thermostable DNA polymerase of *Pyrococcus furiosus* (Pfu) and we have completed a characterization of Pfu DNA polymerase that has resulted in the identification of conserved amino acid residues that affect dNTP binding and utilization. We have mutated these and other residues within Ad pol, and then introduced these mutations into an infectious molecular clone of Ad5. These studies have shown that conservative Ad pol mutations with modest effects on dNTP utilization are compatible with adenovirus replication and recovery of infectious virus (I664V, I664M, M689V, and a S928L/C687S double mutant). In contrast, less conservative mutations and mutations with more profound effects on the dNTP binding efficiency of the adenovirus DNA polymerase give rise to replication-defective viruses. In year 3 (a no-cost extension period), we will test whether these viruses can selectively replicate in, and kill, breast cancer cells.

15. SUBJECT TERMS – Key words or phrases identifying major concepts in the report.
Adenovirus, Conditionally-replicating adenovirus vector, DNA polymerase, dNTP binding, oncolytic virus

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	17	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

The development of effective treatments for breast cancer may require an array of different approaches, including gene therapy-based methods. Oncolytic, conditionally-replicating adenoviruses (CRADs) represent one such method, which can be used either alone or in combination with other treatments (including radio-, chemo- or immuno- therapies). While initial clinical studies of oncolytic CRADs have shown that these vectors are generally safe and well tolerated, additional improvements will be necessary to make oncolytic CRADs more effective. Thus, the key goal of this project is to develop more effective oncolytic CRADs, by using a highly flexible approach that can be readily integrated into, or combined with, other approaches in order to develop an optimal therapeutic product.

BODY

Approved Tasks

The following tasks were outlined in the modified approved statement of work for this grant, that was approved as part of the Year One progress report:

1. **Task One (scope unchanged):** To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess. *Modified Timeline: Will extend in year two (at least to month 18)*.

2. **Task Tw o (scope unchanged):** To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro. *Modified Timeline: Will extend at least to month 24.*

3. **Task Three (unchanged):** To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo. *Original Timeline: months 13-24; Modified Timeline: Will be initiated during a projected no-cost extension period (months 25-36).*

Research Accomplishments associated with the above tasks

Task 1. To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess.

a. Mutation of selected polymerase residues within Ad pol using site-directed mutagenesis.

Construction of Ad5 replication competent viral molecular clones

We have created a series of conservative substitutions at pol residues known to affect dNTP utilization. Mutations include conservative changes at residue 664 (I664V, I664Y) and at residue 690 (Y690F, Y690I, Y690V). All of these mutants have been inserted into the backbone of a replication competent (E1+) Ad5 vector (Task 2).

Continuation of studies on Pfu DNA polymerase as a model for Ad Pol

We continued to use a novel screening assay to identify key residues within Ad pol that will alter dNTP binding and utlization. As noted in our Year One Progress report, this approach relies on the high degree of conservation of the core dNTP binding motifs between Ad pol and the thermostable Family B DNA polymerase from *Pyrococcus furiosus* (Pfu Pol).

Summary of findings on Pfu Pol: We have now completed a careful kinetic characterization of Pfu DNA polymerase. Our results show that Pfu Pol contains sensitive determinants of both dNTP binding and replicational fidelity within the highly conserved Motif A. Site-directed mutagenesis of the Motif A SYLP region revealed that small shifts in side chain volume result in significant changes in dNTP binding affinity, steady state kinetics, and fidelity of the enzyme. Mutants of Y410 show high fidelity in both misincorporation assays and forward mutation assays, but display a substantially higher Km than WT. In contrast, mutations of the upstream residue L409 result in drastically reduced affinity for the correct dNTP, a much higher efficiency of both misincorporation and mismatch extension, and substantially lower fidelity as demonstrated by a PCR-based forward mutation assay. The A408S mutant, however, displayed a significant increase in both dNTP binding affinity and fidelity.

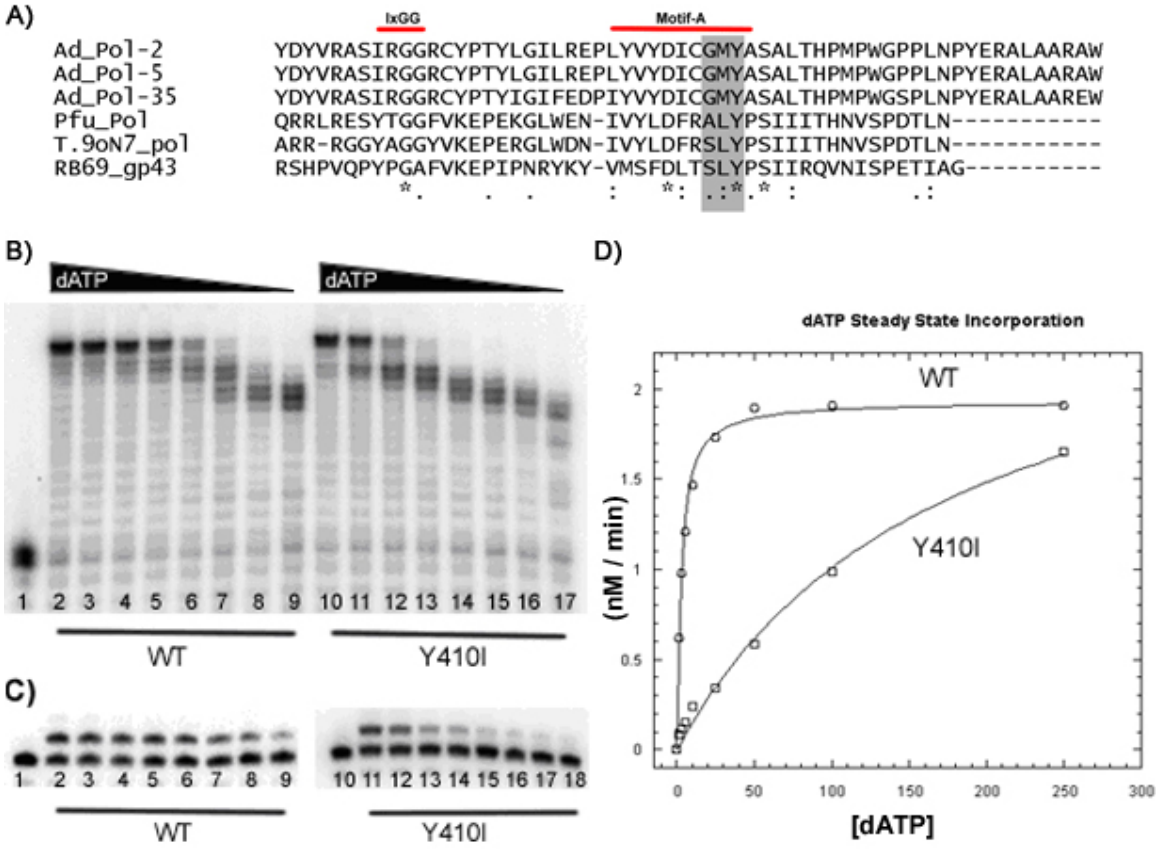
These data show that modulation of Motif A can greatly shift both the steady and pre-steady state kinetics of the enzyme as well as fidelity of Pfu Pol.

Kinetic Analysis of Pfu Pol Motif A Mutants: The central “steric gate” tyrosine (Tyr) or phenylalanine (Phe) in the well-conserved Motif A is responsible for the selective utilization of dNTP versus rNTP substrates by many Pol α family polymerases and reverse transcriptases. This Tyr or Phe residue is presumed to sterically clash with the rNTP 2’ OH and likely participates in a Van der Waals stacking interaction with the sugar moiety of the incoming dNTP substrate. To investigate the role of the

corresponding residue in the functional activity of the Pfu DNA polymerase, we performed site-directed mutagenesis on the Tyr 410 (Y410) residue of Pfu DNA polymerase and its two preceding residues, L409 and A408. To minimize the gross structural effects of these substitutions, we mutated these targeted residues of Motif A to amino acids with physicochemical characteristics similar to the wild type residues. We therefore created the following mutants: Y410L, Y410I, Y410V, L409I, L409V, L409M, L409F, and A408S. Initially, we employed a steady state kinetic screening assay at 55˚ C to evaluate the overall impact of the Motif A mutations on the dNTP concentration-dependent product formation in the polymerase background without 3´-5´ exonuclease activity (D215A substitution). Subsequent pre-steady state kinetic analysis and steady state fidelity assays were then performed with Pfu Pol WT and select mutants to investigate the specific effects of Motif A mutations.

First, in both steady state single and multiple nucleotide incorporation analyses, we observed that mutations at residues 410, 409, and 408 altered incorporation kinetics. Y410I displayed the most significant reductions in dNTP concentration-dependent activity among the mutants tested in both multiple (**Fig.1B**) and single (**Fig.1C**) incorporation reactions. Quantifying the products of a similar single nucleotide extension reaction and fitting them to the Michaelis-Menten equation allowed us to obtain K_m (**Fig.1D and Fig. 2**). Although steady-state kinetics assays do not reveal the specific mechanistic effects resulting from active site mutations, they are useful to screen many mutants and may indicate overall enzyme function or disfunction (i.e. they report the sum of the entire deoxynucleotide incorporation equilibrium).

Figure 1: PFU Steady State Kinetics and dNTP Utilization.

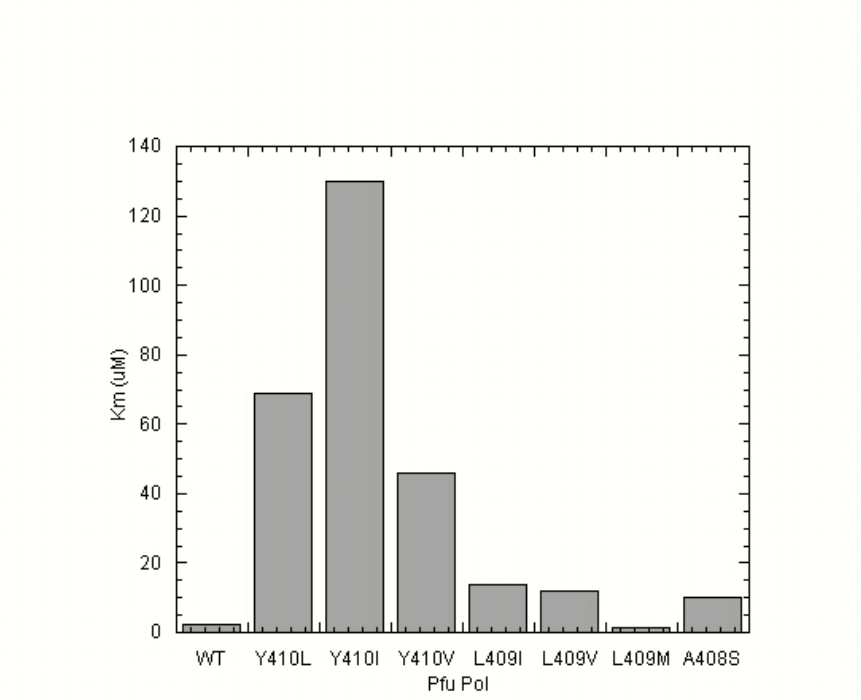


LEGEND. A) Clustal-W multialignment of Adenovirus (serotypes 2,5,35), Pfu Pol, Thermococcus species 9oN7˚ Pol, and RB69 gp43 phage polymerase Motif-A. The specific area of interest to this study is highlighted in grey. "*" represents identical residues, ":" represents conserved substitutions, and "." Represents semi-conserved substitutions B) Multiple nucleotide incorporation reactions were carried out with a 5' end 32P-labeled 23-mer primer annealed to a 40-mer DNA template at 55° C for 5 minutes with varying amounts of all four dNTPs. No enzyme control (lane 1) indicates the position of the 23-mer unextended primer. A representative multi-nucleotide dNTP titration for WT Pfu Pol 3'-5' exo ¬- (lanes 2-9) and Pfu Pol Mutant Y410I (lanes 10-17) is shown; concentrations of dNTP for each set of eight reactions was as follows: (in µM) 250,100, 50, 25, 10, 5, 2.5, 1. C) Representative single nucleotide (dATP) titrations for WT Pfu Pol (lanes 2-9) and Y410I (lanes 11-18) were carried out at 55°C as described in B) except using only dATP. D) Nonlinear regression fit of the Michaelis-Menten equation to the single nucleotide extension performed in duplicate for WT (circles) and Y410I (squares).

Comparison of Pfu Pol WT exo- to ***Y410 mutants revealed a profound defect in dNTP utilization*** at lower substrate concentrations in both multiple nucleotide and single nucleotide extension. We observed a 20 to 61 fold difference in K_m when comparing WT to the Y410 mutants, representing the largest kinetic defect. Mutation of

the L409 residue resulted in a more subtle steady state defect relative to WT, with an approximate 5 fold difference between the WT enzyme versus L409I or L409V (**Fig. 2**). Possibly, alterations of Y410 itself or the neighboring side chains to a less bulky hydrophobic residue reduce or shift the molecular surface available for a Van der Waals interaction with the ribose moiety of an incoming dNTP. In contrast, a conservative substitution of the A408 residue with serine had little effect on steady state kinetic parameters (reflected by only a slight reduction in K_m), although it did result in increased affinity for the incoming dNTP as measured by the presteady state kinetics (**Table 1**). We also constructed and examined additional residue A408 mutants (G, C, and V), and surprisingly found that these mutants had either grossly impaired activity or were completely inactive (data not shown) in contrast to the more conservative A408S mutant (**Fig. 2**). This demonstrates that A408 plays an important structural role in the function of the Motif A loop and is essential for polymerase function.

Figure 2: K_m Values of PFU Pol WT and Mutant proteins.



LEGEND: Steady state single nucleotide reactions were carried out at 55° C and analyzed in duplicate as described in **Figure 1**. The standard deviations are as follows; WT 0.26, Y410L 15.50, Y410I 7.29, Y410V 7.74, l409I 6.51, L409V 0.38, L409M 0.38, and A408S 1.16.

Table 1: Motif-A Mutant Pre-steady State Kinetic parameters (37°C)

Pfu Pol *	K_d (μM) **	k_{pol} (s^{-1})	k_{pol}/ K_d
WT	14.34 ± 2.80	2.18 ± 0.01	0.1520
Y410V	37.73 ± 26.64 (2.42X)	3.73 ± 0.36	0.0988
L409M	305.21 ± 8.49 (21.28X)	9.75 ± 1.21	0.0319
A408S	4.44 ± 2.05	1.88 ± 0.11	0.4234

* all are 3'-5' exonuclease inactive D215A mutation.
** reactions were performed at 37˚C

Based on these results from Pfu DNA polymerase, we constructed a series of mutations at the corresponding residues in the adenovirus polymerase, and then tested their ability to confer a conditionally-replicative phenotype on an adenovirus vector (see **Task 2**).

Mutants constructed included: (1) mutants of the Y690 residue in Ad pol (equivalent to Pfu pol Y410); (2) mutants of the adjacent G688 and M689 residues in Ad pol (equivalent to Pfu pol L409 and A408).

b. Expression of mutated polymerase proteins in insect cells using a baculovirus vector system, and purification of the polymerase from those cells.

Progress:

In order to biochemically characterize Adenovirus polymerase proteins we will need the three virally encoded proteins. These include the Ad5 DNA polymerase, DBP (DNA Binding Protein), and pTP (pre-terminal protein). As a result, the DNA sequence encoding the full length Ad5 DNA polymerase was cloned into the baculovirus pAcgp67A vector and subjected to standard site-directed mutagenesis to create Adenovirus type 5 (Ad5) polymerases containing selected mutations. We also cloned the full length sequence of DBP and pTP into the baculovirus pAcgp67A vector (**Table 3**).

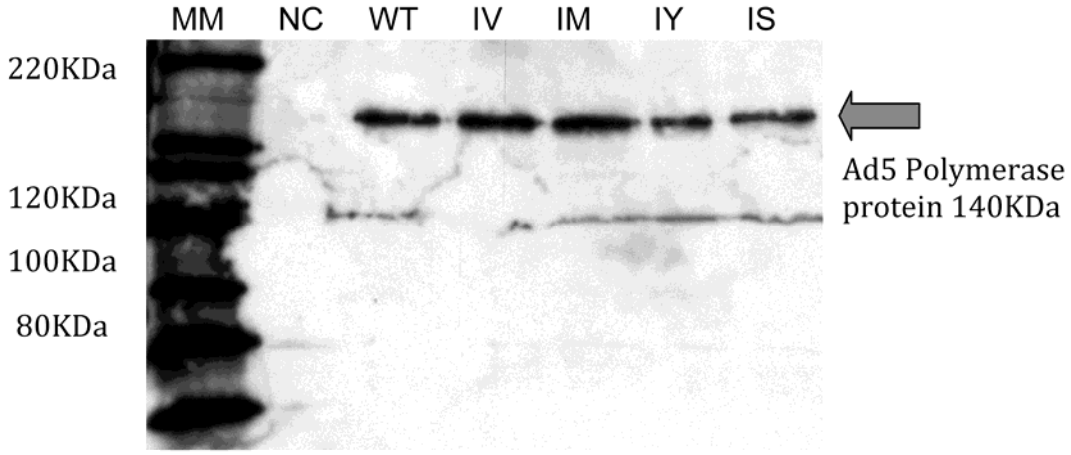
Table 3: Construction of Recombinant Baculovirus and status of construction

Baculovirus Construct	Description of Mutation
I664V Ad5 Pol – pAcgp67A	Protein Production in SF9 insect cells
I664M Ad5 Pol – pAcgp67A	""
I664S Ad5 Pol – pAcgp67A	""
I664Y Ad5 Pol – pAcgp67A	""

WT Ad5 Pol – pAcgp67A	“”
M689V Ad5 Pol – pAcgp67A	Awaiting Transfection
pTP – pAcgp67A	Awaiting Transfection
DBP – pAcgp67A	Under Construction

Expression of the adenovirus proteins I664V, I664M, I664Y, I664S, and WT Ad5 is being carried out in SF9 insect cells (**Figure 3**).

Figure 3: Expression of Ad pol proteins in insect cells



LEGEND: *3µg of recombinant baculovirus DNA was transfected with Cellfectin (Invitrogen) in SF9 insect cells. Three days later the recombinant baculoviruses were collected and used for viral amplification. Expression of Ad5 pol proteins was verified after the second round of viral amplification in SF9 insect cells. Analysis of cell lysates by SDS-PAGE indicated that all of the mutant Ad5 pol proteins are being expressed.* ***MM****, Magic Mark;* ***NC****, Negative Control;* ***WT****, WT Ad5 Pol;* ***IV****, I664V Ad5 Pol;* ***IM****, I664M Ad5 Pol;* ***IY****, I664Y Ad5 Pol;* ***IS****, I664S Ad5 Pol.*

c. Biochemical characterization of the catalytic activity and dNTP binding affinity of the purified polymerase enzymes over a range of different substrate (dNTP) concentrations.

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A & B above)

Task 2. To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro.

a. Mutagenesis of the polymerase gene within full-length, replication-competent Ad5 vector backbones.

Progress: We have created a series of conservative substitutions at pol residues known to affect dNTP utilization, including I664, M689, Y690, S692, R833, R837, N841 and Y844. All of these mutants have been introduced into the backbone of a replication competent (E1+) Ad5 vector. Of the viruses created in this manner, none were viable - with the exception of the I664V, I664M, M689V and S928L/C687S double mutant – all of which were found to be replication-competent. Efforts to characterize the I664V, I664M, and M689V mutantes virus are ongoing; derivation and analysis of a S928L single mutant virus is also underway.

Table 2: Polymerase mutants and current status of virus construction

Mutation	Viral Pol. Motif	Description of Mutation	Progress
I664V	IxGG Motif	Expected to disrupt interaction of template DNA and polymerase	**Replication-competent viral molecular clone**
I664M	IxGG Motif	‘’	**Replication-competent viral molecular clone**
S928L/C687S	Motif A	Expected to disrupt polymerase-dNTP interaction	**Replication-competent viral molecular clone**
M689V	Motif A	‘’	**Replication-competent viral molecular clone**
I664S	*IxGG Motif*	*Expected to interaction of template DNA and polymerase*	*Non-viable virus mutant*
I664Y	*IxGG Motif*	“”	*Non-viable virus mutant*
GG666/7AA	*IxGG Motif*	“”	*Non-viable virus mutant*
M689I	*Motif A*	*Identified by screening of Pfu DNA polymerase; expected to disrupt polymerase-dNTP interaction*	*Non-viable virus mutant*
G688S	*Motif A*	“”	*Non-viable virus mutant*
Y690A	*Motif A*	“”	*Non-viable virus mutant*
Y690F	*Motif A*	“”	*Non-viable virus mutant*
Y690I	*Motif A*	“”	*Non-viable virus mutant*
Y690V	*Motif A*	“”	*Non-viable virus mutant*
S692Y	*Motif A*	“”	*Non-viable virus mutant*
R833T	*Motif B*	*Expected to disrupt dNTP binding affinity*	*Non-viable virus mutant*
Y844S	*Kx3NSxYG Motif*	*Expected to disrupt interaction of template DNA and polymerase*	*Non-viable virus mutant*
K837N	*Kx3NSxYG Motif*	“”	*Non-viable virus mutant*
N841E	*Kx3NSxYG Motif*	*Expected to disrupt interaction of template DNA and polymerase*	*Non-viable virus mutant*
R665K	IxGG Motif	“”	Under Construction
N841Y	Kx3NSxYG Motif	“”	Under Construction

b. Generation, purification and titration of Ad5 vector stocks.

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A above). This will task will performed in the no-cost extension period (year three).

c. Quantitative analysis of vector replication and host cell cytolysis in a panel of breast cancer cells lines and in primary human mammary epithelial cells.

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A above). This will task will performed in the no-cost extension period (year three).

Task 3. To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo.

Progress: This Task was previously scheduled for the no-cost extension period (year three). There is no change in this anticipated timeline.

Modified Statement of Work

In light of the need to extend the continuing unanticipated complexity of Task 1 (noted above), we therefore propose a **modified Statement of Work**, as outlined below (note that the major change here is a revision of the timeline, not the actual work).

4. **Task One (scope unchanged):** To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess. *Previous timeline: will extend into year two (at least month 18); Modified timeline: Will extend into year three (at least to month 30)*.

5. **Task Tw o (scope unchanged):** To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro. *Previous timeline: Will extend at least to month 24; Modified timeline: Will extend into year three (possibly up to month 36)*

6. **Task Three (unchanged):** To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo. *Previous timeline: Will be initiated during a projected no-cost extension period (months 25-36); Modified timeline: No change*

IMPORTANT NOTE: Because of the fact that it will be essential to derive adenovirus vectors that selectively replicate in low dNTP environments (such as tumor cells) before we can proceed with in vivo experiments, the in vitro Tasks (Tasks 1, 2) must remain our major focus. This is a scientific necessity. As noted in our Year One report, should progress prove slower than anticipated, it is conceivable that we may not initiate the in vivo studies (Task Three). Should that prove to be the case, we will consider a future grant application to undertake this work.

KEY RESEARCH ACCOMPLISHMENTS

Current Project Year

- We have identified an important surrogate for the adenovirus DNA polymerase (Ad pol): the thermostable DNA polymerase of Pyrococcus furiosus (Pfu) and we have completed a characterization of Pfu DNA polymerase that has resulted in the identification of conserved amino acid residues that affect dNTP binding and utilization.

- Kinetic characterization of Pfu DNA polymerase has shown that this enzyme contains sensitive determinants of both dNTP binding and replicational fidelity within the highly conserved Motif A - a region that is conserved in the Ad pol. Site-directed mutagenesis of the Motif A SYLP region revealed that small shifts in side chain volume result in significant changes in dNTP binding affinity, steady state kinetics, and fidelity of the enzyme. Mutants of Y410 show high fidelity in both misincorporation assays and forward mutation assays, but display a substantially higher K_m than WT. In contrast, mutations of the upstream residue L409 result in drastically reduced affinity for the correct dNTP, a much higher efficiency of both misincorporation and mismatch extension, and substantially lower fidelity as demonstrated by a PCR-based forward mutation assay. The A408S mutant, however, displayed a significant increase in both dNTP binding affinity and fidelity. These data show that modulation of Motif A can greatly shift both the steady and pre-steady state kinetics of the enzyme as well as fidelity of Pfu Pol.

- We have mutated the conserved residues identified above, and other residues within Ad pol, and then introduced these mutations into an infectious molecular clone of Ad5. These studies have shown that conservative Ad pol mutations with modest effects on dNTP utilization are compatible with adenovirus replication and recovery of infectious virus (I664V, I664M, M689V, and a S928L/C687S double mutant). In contrast, less conservative mutations and mutations with more profound effects on the dNTP binding efficiency of the adenovirus DNA polymerase give rise to replication-defective viruses. *A total of 18 Ad pol mutations have now been analyzed for their effects on virus replication using this approach; studies of 2 additional mutants are in progress.*

- We have subcloned the genes encoding the adenovirus DNA polymerase (pol), terminal protein (pTP) and DNA-binding protein (DBP) into a baculovirus vector and shown that we can obtain robust expression of Ad pol in insect cells.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: See Bibliography.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportuniti es applied for and/or received on experiences/training supported by this award:

Research training was provided for the following persons during the present project year:

Ms. Cristina Capella-Gonzalez, a Ph.D. trainee in the Dewhurst laboratory. Ms. Capella is presently conducting her thesis research at the University of Rochester Medical Center, under the supervision of Dr. Dewhurst. She is a member of an under-represented minority group.

CONCLUSIONS

The conclusions which can be drawn from the second year of our experiments are as follows:

1. We have identified an important surrogate for the adenovirus DNA polymerase (Ad pol): the thermostable DNA polymerase of Pyrococcus furiosus (Pfu). Kinetic characterization of Pfu DNA polymerase has shown that this enzyme contains sensitive determinants of both dNTP binding and replicational fidelity within the highly conserved Motif A - a region that is conserved in the Ad pol. Site-directed mutagenesis of the Motif A SYLP region revealed that small shifts in side chain volume result in significant changes in dNTP binding affinity, steady state kinetics, and fidelity of the enzyme. Mutants of Y410 show high fidelity in both misincorporation assays and forward mutation assays, but display a substantially higher K_m than WT. In contrast, mutations of the upstream residue L409 result in drastically reduced affinity for the correct dNTP, a much higher efficiency of both misincorporation and mismatch extension, and substantially lower fidelity as demonstrated by a PCR-based forward mutation assay. The A408S mutant, however, displayed a significant increase in both dNTP binding affinity and fidelity. These data show that modulation of Motif A can greatly shift both the steady and pre-steady state kinetics of the enzyme as well as fidelity of Pfu Pol.

2. We have examined the effect of Ad pol mutations on virus replication, by introducing mutations in conserved Ad pol residues into the context of a replication-competent Ad5 virus genome. These studies have revealed that conservative Ad pol mutations with modest effects on dNTP utilization are compatible with adenovirus replication and recovery of infectious virus (I664V, I664M, M689V, and a S928L/C687S double mutant). In contrast, less conservative mutations and mutations with more profound effects on the dNTP binding efficiency of the adenovirus DNA polymerase give rise to replication-defective viruses.

3. We have determined that the adenovirus DNA polymerase (pol) can be efficiently expressed in insect cells using a baculovirus vector system.

"So What Section"

Our data advance the goals of this grant application, and bring us closer to being able to test our underlying hypothesis, that adenovirus vectors with mutated DNA polymerase genes may represent a powerful new approach for permitting selective vector replication in breast cancer cells, thereby killing breast cancer cells while sparing normal tissue.

BIBLIOGRAPHY (PUBLICATIONS)

No publications were generated in the project year covered by this report

One manuscript is presently under review. Once this manuscript appears in print, a copy will be shared with the funding agency:

- Edward M. Kennedy, Christopher Hergott, Stephen Dewhurst and Baek Kim. The Mechanistic Architecture of the Thermostable Pyrococcus Furiosus Family B DNA Polymerase Motif A and its Interaction with dNTP Substrate. Biochemistry, under review, 2009.